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Gamma Interferon-Induced Nitric Oxide Production Reduces *Chlamydia trachomatis* Infectivity in McCoy Cells

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McCoy cells, murine-derived cells commonly used for propagation of chlamydiae, were found to be efficient producers of nitric oxide (NO) when primed with murine gamma interferon (IFN-γ) and then exposed to the second signals provided by *Escherichia coli* lipopolysaccharide, human interleukin-1α, murine tumor necrosis factor α, or *Chlamydia trachomatis* type H. Murine recombinant IFN-γ over a range of 0 to 50 U/ml inhibited infectivity of *C. trachomatis* type H in a dose-dependent fashion in McCoy cells while simultaneously inducing NO production. Quantitation of infectious chlamydia progeny remaining in McCoy cells 48 or 72 h postinfection revealed that IFN-γ-primed McCoy cells reduced chlamydial inclusion-forming units (expressed as units per milliliter) by 4 $\log_{10}$ units at higher IFN-γ concentrations (50 U/ml) compared with control values. The magnitude of this antichlamydial effect was directly related to increased synthesis of NO, the production of which was IFN-γ dose dependent. The antichlamydial effects of IFN-γ were blocked in a dose-dependent manner by the addition of *N*-guanidino-monomethyl L-arginine (MLA), an inhibitor of nitric oxide synthesis. These results suggest that although IFN-γ priming of McCoy cells is required for antichlamydial activity, nitric oxide is a necessary effector molecule involved in the mechanism(s) of IFN-γ-induced inhibition of chlamydial proliferation in this murine cell line. The ability to block the potent antichlamydial effects of IFN-γ by inhibition of a specific enzyme, nitric oxide synthase, may give insights into mechanisms by which IFN-γ and perhaps other cytokines are able to control proliferation of chlamydiae and other intracellular pathogens.

Previous investigators have shown that gamma interferon (IFN-γ) from both human and murine sources exerts a profound inhibitory effect on chlamydial growth and viability (4–6, 13, 14, 28, 39, 42–44). While IFN-γ induces at least one antichlamydial effector pathway in human cell lines through altered tryptophan metabolism (5, 36, 40), other mechanisms are responsible for IFN-γ effects in murine cell lines (12, 36). One possible mechanism through which IFN-γ-dependent antichlamydial activity is mediated is induction of nitric oxide. Nitric oxide has potent cytotoxic effects on the facultative intracellular parasite *Cryptococcus neoformans* (20), shistosomula of *Schistosoma mansoni* (30), intracellular amastigotes of *Leishmania major* (21, 22, 34), and intracellular trophozoites of *Toxoplasma gondii* (1a). IFN-γ can prime or direct subsequent cellular enzymatic pathways to produce nitric oxide when macrophages or nonmacrophage somatic cells are exposed to a second signal such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), or interleukin-1 (IL-1) (2, 33). Nitric oxide is synthesized by the enzyme nitric oxide synthase, which uses L-arginine as the sole substrate to eventually produce the end metabolic products of nitrate, nitrite, and citrulline (2, 24–27, 29). Mammalian cells have both low-level constitutive nitric oxide synthase activity and the potential for cytokine-inducible high-output nitric oxide synthase activity (2). Nitric oxide synthesis in murine cell culture is competitively inhibited by the L-arginine analog *N*-guanidino-monomethyl L-arginine (MLA), with no effect on cell proliferation (24, 27). The availability of a specific inhibitor (MLA) for the cytokine-inducible enzyme which produces nitric oxide allowed testing of the hypothesis that IFN-γ mediates its antichlamydial effect in murine cells by inducing synthesis of nitric oxide.

MATERIALS AND METHODS

Growth of chlamydiae and maintenance of cell lines. *Chlamydia trachomatis* type H and McCoy cells were obtained from American Type Culture Collection (Rockville, Md.). McCoy cells were grown in Eagle's minimal essential medium (EMEM; Flow Laboratories, McLean, Va.) supplemented with 10% heat-inactivated fetal bovine serum (obtained from Hyclone, Salt Lake City, Utah) and streptomycin (50 μg/ml) at 35°C in a 5% CO_2 humidified atmosphere. Cells were regularly tested and found to be free of contamination with mycoplasmas. Chlamydia stock used for these experiments was grown on McCoy cell monolayers in the presence of cycloheximide (2 μg/ml) and streptomycin (50 μg/ml) in 75-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark). Flasks were inoculated with sufficient numbers of elementary bodies (EBs) to produce inclusions in 80 to 90% of McCoy cells in monolayers. Centrifugation at 1,000 × *g* for 30 min at 35°C was performed to facilitate infection. The infected monolayers were incubated at 35°C in a 5% CO_2 atmosphere for 60 to 72 h prior to purification. The majority of cells were intact and contained large inclusions visible by inverted microscopy.

Purification of EBs and RBs. The following method was adapted from the method of Caldwell et al. (7). Flasks were freeze-thawed and then sonicated in a water bath sonifier (Bransonic 12; Branson, Shelton, Conn.) for 10 min. Heparin was added to a final concentration of 20 U/ml, and sonication was repeated. Cell debris was pelleted by centrifugation at 1,000 × *g* for 7 min and discarded. Chlamydiae (EBs and reticulate bodies [RBs]) from the supernatant were pelleted

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by centrifugation at 16,000 × *g* for 20 min. The resultant pellet was gently suspended in SPG (250 mM sucrose, 10 mM sodium phosphate, 5 mM glutamic acid; pH 7.2) and digested with DNase I (0.1 mg/ml) and RNase A (0.1 mg/ml) in the presence of 10 mM $MgCl_2$ for 30 min at room temperature. Chlamydiae were purified by centrifugation at 70,000 × *g* through 30% (vol/vol) Hypaque (Winthrop Pharmaceuticals, New York, N.Y.) (7) in an SPG cushion resulting in a pellet of EBs and RBs. The pellet was gently resuspended in SPG and centrifuged through a step gradient of 30% (vol/vol)–35% (vol/vol)–40% (vol/vol) Hypaque. The SPG–30% Hypaque layer was discarded (residual cellular debris), RBs were collected at the 30–35% Hypaque interface, and EBs were collected at the 35–40% Hypaque interface. EBs and RBs were washed in SPG and resuspended in sterile modified standard reaction medium (23) containing 100 mM $TrisCl_2$, 50 mM KCl, and 5 mM $MgCl_2$ plus 10% dimethyl sulfoxide and frozen at −70°C in 50-μl aliquots until used. Inclusion-forming unit (IFU) determinations for EB aliquots were done as described below.

Cytokine priming of McCoy cells to determine their potential to produce nitric oxide. Recombinant murine IFN-γ (2.3×10^7 U/ml) and murine tumor-necrosis factor alpha (TNF-α) (2.8×10^7 U/ml) were obtained from Genentech Inc., South San Francisco, Calif. Human IL-1α was provided by Hoffmann-La Roche Inc., Nutley, N.J. *Escherichia coli* LPS was obtained from Associates of Cape Cod, Woods Hole, Mass. MLA was obtained from Chem Biochem Inc., Salt Lake City, Utah, and was prepared freshly for each experiment. Experiments were performed in 24-well tissue culture plates (Nunc) with confluent McCoy cells (5×10^5 per well) in EMEM supplemented with 1% fetal bovine serum. Experiments were first done with cytokines and LPS individually and in combination to determine whether McCoy cells could produce nitric oxide after IFN-γ priming. McCoy cell monolayers (5×10^5 per well) in triplicate received individual treatment with either medium as a control, IFN-γ (20 U/ml), or TNF-α (50 U/ml). Nitrite was measured 72 h post-cytokine treatment (see "Determination of nitrite and nitrate levels" below). Additional McCoy cell monolayers (5×10^5 per well) in triplicate received the following combination treatments: (i) IFN-γ (20 U/ml) plus LPS (20 ng/ml), (ii) IFN-γ (20 U/ml) plus IL-1α (10 U/ml), (iii) IFN-γ (20 U/ml) plus TNF-α (25 U/ml), and (iv) IFN-γ (20 U/ml) plus IL-1α (10 U/ml) and TNF-α (25 U/ml). Nitrite present in the supernatant 72 h after treatment was measured as described below.

Cytokine priming prior to chlamydial infection. All experiments involving chlamydial infection following cytokine priming were done in duplicate and included two infected and two uninfected wells per cytokine treatment. IFN-γ (0 to 50 U/ml) was added to cells 24 h prior to infection with EBs of *C. trachomatis* type H unless otherwise specified. MLA was added to the culture medium at the time of chlamydial infection at a final concentration of 0, 0.2, or 0.8 mM. The L-arginine concentration in EMEM was 0.6 mM. Some experiments utilized the addition of excess L-arginine at 2.4 mM (final concentration) at the time of IFN-γ priming to determine whether addition of this substrate of NO synthase either enhanced or inhibited recovery of infectious progeny 48 and 72 h postinfection. Experiments were done with a multiplicity of infection (MOI) of 1 (1 IFU: 1 McCoy cell) unless specified otherwise. Uninfected monolayers received a sham infection with the same volume (about 5 μl) of the standard reaction medium used for freezing chlamydiae, which contained 10% dimethyl sulfoxide (0.5 μl). After inoculation with chlamydiae or sham infection, the plates were centrifuged at 1,000 × *g* for 30 min at 35°C and then allowed to incubate at 37°C in a 5% CO_2 humidified atmosphere.

Plates were allowed to incubate for either 24, 48, or 72 h. Supernatants were removed at each of these time points, and samples were frozen at −70°C until nitric oxide metabolites were assayed. Cell viability was estimated by trypan blue exclusion assay. Infected monolayers at both 48 and 72 h postinfection and supernatants remaining at 48 and 72 h were assayed for chlamydial infectivity as described below.

Quantitation of chlamydial infectivity. Infectivity of EBs present in either the purified EB fractions used as inocula (see above) or in McCoy cell monolayers at 48 or 72 h after chlamydial infection or in supernatants 48 or 72 h postinfection were quantitated by previously described techniques (39). (This procedure quantified the infectious progeny originating from the initial inoculum [MOI = 1] and was measured at 48 and 72 h postinfection.) Briefly, infected monolayers were held at −70°C until subjected to a thaw cycle and then addition of 1 ml of EMEM, followed by manual disruption of the monolayers by scraping and repeated pipetting. This suspension, which had no observable intact cells, was further subjected to sonication for 1 min in a Bransonic 12 water bath sonicator (Branson) to facilitate disaggregation of chlamydiae from cell debris. Purified EBs which were used as inocula for these experiments were subjected to a 1-min sonication step prior to the dilution steps described below. Supernatants taken from infected monolayers at 48 or 72 h were assayed in a similar fashion. Duplicate serial 10-fold dilutions of these suspensions were used to inoculate confluent McCoy cell monolayers supplemented by 5% heat-inactivated fetal bovine serum in EMEM in the presence of 2-μg/ml cycloheximide in 96-well tissue culture plates. The plates were centrifuged at 1,000 × *g* for 30 min, incubated for 72 h, and then stained with Jones iodine (10) to detect inclusion bodies of chlamydiae by inverted microscopy at 200× magnification. Total numbers of chlamydial inclusions in the wells containing the most diluted observable chlamydial inoculation were determined. This method was highly reproducible when known inocula of purified EBs as well as suspensions from infected cells derived as described above were used. We confirmed the reproducibility of the sensitivity and specificity of this method by using fluorescein isothiocyanate-labelled antichlamydial monoclonal antibody according to the manufacturer's directions (Pathfinder; Kallestad, Chaska, Minn.) in identically infected confluent McCoy cell monolayers on removable glass coverslips in tissue culture vials which were then examined by fluorescence microscopy. This latter method is in current use. Chlamydial infectivity was expressed as IFU per milliliter of the original inoculum. In the 24-well experiments described above in "Cytokine priming prior to chlamydial infection," IFU per milliliter is equal to total infectious chlamydiae per well present at 72 h after infection.

Determination of nitrite and nitrate levels. *E. coli* nitrate reductase was prepared according to previously described methods (3). Equal volumes (50 μl of each) of *E. coli* nitrate reductase suspension and supernatants from control and infected McCoy cells were mixed and incubated at 37°C for 60 min, with reduction of nitrate to nitrite as described by Granger et al. (20). The concentration of nitrite was determined by the Greiss reaction according to a previously described microassay (16). After incubation, these reaction mixtures were subjected to centrifugation at 1,000 × *g* for 10

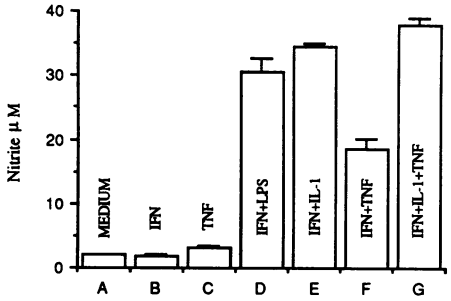

FIG. 1. Effects of cytokines and LPS on nitrite production in McCoy cells. Confluent McCoy cell monolayers (5×10^5 cells per well) were untreated (medium control) (A) or treated with IFN-γ alone (20 U/ml) (B), TNF-α alone (50 U/ml) (C), IFN-γ (20 U/ml) plus LPS (10 ng/ml) (D), IFN-γ (20 U/ml) plus IL-1α (10 U/ml) (E), IFN-γ (20 U/ml) plus TNF-α (25 U/ml) (F), or IFN-γ (20 U/ml) plus IL-1α (10 U/ml) and TNF-α (25 U/ml) (G). Values are mean total concentrations of nitrite ±2 standard errors of the mean (SEM) (95% confidence intervals) present in supernatants at 72 h post-cytokine-LPS treatment from three determinations of a representative experiment. Differences in experimental groups were termed significant when values fell outside the error bars ($P < 0.05$).

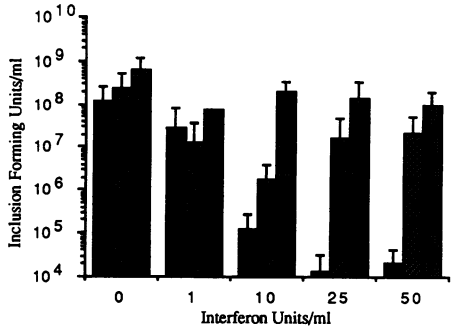

FIG. 2. Effects of IFN-γ priming and MLA on chlamydial infectivity 72 h postinfection with an MOI of 1. McCoy cell monolayers were primed with IFN-γ (0 to 50 U/ml) 24 h prior to infection with *C. trachomatis* type H. At the time of infection, McCoy cell monolayers received no MLA as a control (black bars) or 0.2 mM (hatched bars), or 0.8 mM (stippled bars) MLA. The concentration of L-arginine in the medium was 0.6 mM. Values are mean IFU ±2 SEM (95% confidence intervals) for three experiments done in duplicate except for one experiment with IFN-γ at 1 U/ml. Differences in experimental groups were termed significant when values fell outside the error bars ($P < 0.05$).

min, and 50 μl of supernatant was then removed and mixed with 100 μl of the Greiss reagent [1% sulfanilamide in 30% acetic acid–0.1% *n*-(1-naphthyl) diethylene diamine dihydrochloride in 60% acetic acid] (1:1), which resulted in an immediate color change in the presence of nitrite. A_{480} was measured in a Beckman DU-65 spectrophotometer. Concentrations were calculated from a linear standard curve by using sodium nitrite at concentrations between 0 and 100 μM which was treated identically to the samples above. Nitrite concentration was determined for each pair of supernatants before and after chemical reduction of nitrate by adding 50 μl of supernatant to 100 μl of Greiss reagent. Nitrate concentration was then calculated as [nitrate] = [nitrite (reduced sample)] − [nitrite (nonreduced sample)]. All samples were done in triplicate, and standard errors were determined.

RESULTS

Production of nitric oxide in McCoy cells following cytokine stimulation. Priming of McCoy cells with IFN-γ or TNF-α alone did not result in significant production of NO compared with that of medium controls (Fig. 1). However, IFN-γ-primed McCoy cells which were treated with a second signal of *E. coli* LPS, IL-1α, or TNF-α were efficient producers of nitrite, one of the end products of NO synthase action on L-arginine, as shown in Fig. 1. *E. coli* LPS and IL-1 appeared to be more effective second signals than TNF-α in McCoy cells. MLA blocked nitrite production induced by these second signals (data not shown).

IFN-γ effects on chlamydial infectivity and anti-IFN effects of MLA. Priming of McCoy cell monolayers with IFN-γ 24 h prior to infection with *C. trachomatis* type H at an MOI of 1 resulted in an IFN dose-dependent decrease in chlamydial progeny infectivity (Fig. 2, black bars), with a decrease in chlamydial progeny infectivity of 4 $\log_{10}$ units at 72 h over the range of 0 to 50 U of IFN-γ per ml. This IFN effect was progressively inhibited with increasing doses of MLA in the culture medium, with partial inhibition at 0.2 mM MLA (Fig. 2, hatched bars) and almost complete inhibition at 0.8 mM MLA (Fig. 2, stippled bars). Virtually all chlamydial infectivity was cell associated, as expected. Supernatants from these infected monolayers were not found to have significant numbers of IFU (≤1 IFU/ml). We also measured IFU produced by chlamydial progeny present at 48 h postinfection, with virtually identical results (data not shown).

Examination of McCoy cell monolayers at 48 and 72 h postinfection using fluorescent monoclonal antibody staining indicated that IFN-γ treatment produced a marked reduction in both the size and the number of chlamydial inclusions seen at both 48 and 72 h postinfection. MLA appeared to partially reverse the antichlamydial effect of IFN-γ so that inclusions were about 40% more numerous and were clearly larger than those seen in IFN-γ-treated monolayers (data not shown).

Addition of excess L-arginine (2.4 mM) to reaction mixtures receiving 0 to 50 U of IFN-γ per ml did not alter the antichlamydial effects of IFN-γ, to either significantly increase or decrease recovery of infectious progeny. However, addition of excess L-arginine did partially reverse MLA inhibition of NO synthesis in IFN-γ-primed McCoy cells (nitrite concentration ranging from 10.7 ± 0.4 μM with 0.8 mM MLA treatment to 16.7 ± 1.5 μM with 0.8 mM MLA and 2.4 mM L-arginine treatment) measured at 48 h postinfection. As the ability of McCoy cells to produce NO was restored by this increased amount of L-arginine, we observed a reduction of 3 $\log_{10}$ units in recoverable chlamydial progeny (IFU) present at 48 h postinfection (5.3×10^{10} IFU/ml with 0.8 mM MLA versus 1.9×10^7 IFU/ml with 0.8 mM MLA plus 2.4 mM L-arginine).

Trypan blue exclusion was used to estimate cell viability of infected and uninfected McCoy cell monolayers at 24, 48, and 72 h post-chlamydial inoculation. Viability was >95% at all time points for uninfected cells primed with IFN-γ alone or in combination with MLA. Viability of infected cells was approximately 95% through 48 h, but at 72 h cell viability decreased to approximately 50% but was similar in all infected treatment groups.

Nitric oxide synthesis associated with reduction of chlamydial infectivity. The ability of IFN-γ to reduce chlamydial infectivity was paralleled by an increase in NO synthesis, represented by nitrite-nitrate in the culture supernatants. MLA inhibited NO synthesis in a dose-dependent fashion (Fig. 3). McCoy cells infected with chlamydiae in the ab-

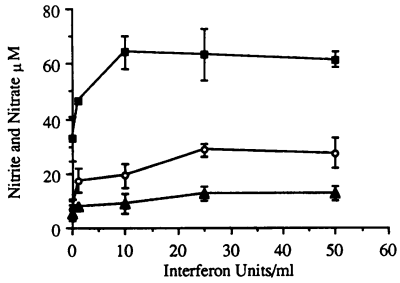

FIG. 3. Effects of MLA and IFN-γ priming on production of nitric oxide metabolites in McCoy cell monolayers infected with *C. trachomatis* type H at an MOI of 1. Total concentrations of nitrite plus nitrate, expressed as micromoles per liter present in supernatants at 72 h postinfection, from cultures treated with no MLA as a control (solid squares) or with 0.2 mM (open circles) or 0.8 mM (solid triangles) MLA are shown at IFN-γ concentrations ranging from 0 to 50 U/ml. Values are mean total concentrations of nitrite plus nitrate ±2 SEM (95% confidence intervals) for three experiments done in duplicate except for one experiment with IFN-γ at 1 U/ml. Differences in experimental groups were termed significant when values fell outside the error bars ($P < 0.05$).

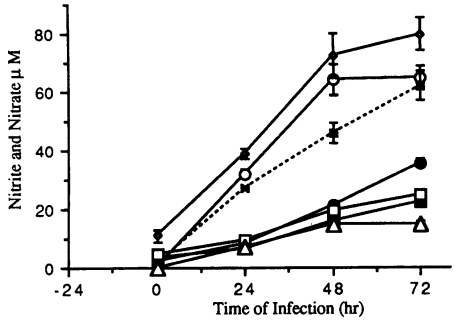

FIG. 4. Effect of timing of IFN-γ dose on NO synthesis in McCoy cells relative to chlamydial infection. IFN-γ (10 U/ml) was added at various time points relative to chlamydia infection (zero time) as indicated. IFN-γ remained in the medium for the duration of the experiments except for the treatment at −24 h plus a wash, in which IFN-γ was added 24 h prior to infection, with removal and replacement of the medium immediately prior to infection. Experimental groups were treated with IFN at −24 h plus a wash (open circles), −24 h (open diamonds), 0 h (dotted line), 24 h (closed circles), or 48 h (closed squares) or were uninfected with no added IFN-γ (open squares) or uninfected with IFN-γ added at −24 h (open triangles). Supernatants were collected at the time points indicated and assayed for nitrite and nitrate. Values are mean total concentrations of nitrite plus nitrate (expressed as micromoles per liter) ±2 SEM (95% confidence intervals) from four determinations of a representative experiment. Differences in experimental groups were termed significant when values fell outside the error bars ($P < 0.05$).

sence of MLA (Fig. 3, closed squares) synthesize NO, which reached a plateau when IFN-γ (10 U/ml) was present. When 0.2 mM MLA was added to the culture medium, there was a significant decrease in the ability of McCoy cells to synthesize NO (Fig. 3, open circles), and this was further diminished at 0.8 mM MLA (Fig. 3, closed triangles). In the absence of chlamydial infection, NO production was not affected by increasing concentrations of IFN-γ in the medium and remained at baseline levels (data not shown). Total nitric oxide synthesis at 48 and 72 h was inversely related to measured recoverable chlamydial IFU obtained at those times. The inhibition of NO synthesis was found to be MLA dose dependent.

Effect of timing of IFN-γ dose on NO production. The time at which IFN-γ was added to infected McCoy cells was important in determining the amount and rate of NO synthesis. As seen in Fig. 4, IFN-γ (10 U/ml) added 24 h prior to, or coincident with, chlamydial infection (MOI of 1) was associated with maximal NO production. This effect reached a peak 72 h after exposure to IFN-γ, regardless of whether IFN-γ remained in the medium throughout the experiment or was removed at the time of infection. When IFN-γ was added 24 h or later postinfection, NO production was similar to that seen in McCoy cells with chlamydia infection alone. Uninfected controls primed with IFN-γ showed minimal baseline NO production. The rate of NO production appeared to be relatively constant over 72 h within each treatment group, with the possible exception of the two −24-h IFN-γ groups, in which NO production appeared to plateau at 48 h postinfection.

DISCUSSION

C. trachomatis, an obligate intracellular parasitic bacterium, is the leading cause of preventable blindness in the world (31), as well as the leading cause of sexually transmitted disease in the United States (8). Because infection with this organism does not confer protective immunity, understanding host defense mechanisms which eradicate or impede the growth of this parasite may prove useful in designing effective means of prevention. Natural defense mechanisms such as cytokine-mediated killing of intracellular pathogens are important immunologic means which maintain the balance of the host-parasite relationship in favor of the host. IFN-γ priming of both macrophage and nonmacrophage cell lines is an example of one such host defense mechanism which has known potent antichlamydial activity (4–6, 13, 14, 28, 39, 42–44). While chlamydial specificity for mucosal tissue may be related to specific host cell-parasite interactions, it may in part be related to relative deficiency of IFN-γ production in mucosal tissues. Immunologic studies in murine models indicate that lymphocytes which are found in lymph nodes draining mucosal surfaces are consistently low-level producers of IL-2 and IFN-γ, in contrast to the lymph nodes draining skin (10a, 11). It would be advantageous to this pathogen to establish its primary sites of infection in tissues with low levels of basal local IFN-γ production.

While at least part of the observed IFN-γ antichlamydial effect in human cells is dependent on enzymatic degradation of tryptophan by indoleamine 2,3-dioxygenase (reversible by addition of extracellular tryptophan) (5, 36, 40), antichlamydial IFN-γ effects are not mediated by this mechanism in murine-derived cell lines (12, 36).

In our investigations of the antichlamydial role of IFN-γ in McCoy cells, we established that McCoy cells were efficient producers of nitric oxide when primed with IFN-γ and exposed to second signals such as IL-1, *E. coli* LPS, and TNF-α (Fig. 1) or chlamydiae (Fig. 3). To our knowledge, this is the first report which details nitric oxide production by McCoy cells. Previous investigators determined that other nonmacrophage murine cell lines are efficient producers of NO (2), and our results were not unexpected. It appears that for optimal production of NO in McCoy cells, IFN-γ priming must precede or at least be coincident with chlamydial infection (Fig. 4). It appears that maximal inducible NO synthesis in McCoy cells occurs even if IFN-γ is removed prior to chlamydial infection. While we determined that

McCoy cells primed with IFN-γ 24 h prior to infection with chlamydiae were efficient producers of NO, it will be important to determine how far prior to chlamydial infection IFN-γ priming of McCoy cell will induce NO production and produce antichlamydial effects. This knowledge will further our understanding of cytokine-induced defense mechanisms for control of intracellular parasitism. The finding that McCoy cells synthesize NO efficiently under these conditions allowed us to test the hypothesis that IFN-γ induces its antichlamydial activity in McCoy cells through the effector molecule nitric oxide.

Inducible nitric oxide production following stimulation of cells with IFN-γ appears to be a conserved mechanism for cells to control proliferation of other intracellular pathogens, such as *T. gondii* (1), *Plasmodium yoelli* (37), and *Mycobacterium tuberculosis* (9, 15). Both macrophages and nonmacrophage cell lines are capable of high-level production of NO when they receive the appropriate sequence of cytokine stimulation (2, 16, 18–20, 24, 27). Cells which are primed with IFN-γ and are subsequently exposed to second signals such as IL-1, TNF-α, or LPS are able to generate high levels of NO. This sequence of signalling results in induction of nitric oxide synthase, which produces NO by oxidative deimination of one of the terminal guanidino nitrogens of L-arginine. This enzyme is inhibited by the L-arginine analog MLA (24, 27).

Our data indicate that IFN-γ-induced nitric oxide production is required for antichlamydial activity in IFN-γ-treated McCoy cells. The 4-log-unit decrease in infectivity of chlamydial progeny remaining 72 h postinfection over the range of IFN-γ used (0 to 50 U/ml) shown in Fig. 2 was accompanied by a corresponding IFN-γ dose-dependent rise in NO as shown in Fig. 3. Chlamydial IFU present at 48 h postinfection revealed a virtually identical pattern of chlamydial growth inhibition and NO production (data not shown). We demonstrated that MLA, a specific competitive inhibitor of nitric oxide synthase activity, progressively inhibited in a dose-response manner the antichlamydial activity of IFN-γ (Fig. 3). In fact, chlamydiae appear to multiply equally well in the presence of IFN-γ and in its absence, if nitric oxide synthesis is blocked by MLA. Although IFN-γ is essential to establish antichlamydial activity in this model, our data show that NO must be synthesized to effect this activity and NO appears to be a final effector molecule of IFN-γ-mediated antichlamydial activity in McCoy cells.

In experiments in which we supplemented IFN-treated cells with excess L-arginine (2.4 mM) in addition to the normal concentration in EMEM (0.6 mM), we found no increase in progeny recovered at either 48 or 72 h when McCoy cells were primed with IFN-γ. In monolayers primed with IFN-γ and treated with 0.8 mM MLA which were supplemented with excess L-arginine, a 3-$\log_{10}$-unit decrease in progeny recovery was noted. This reduction in recovery of infectious progeny in the presence of excess L-arginine was accompanied by a rise in nitrite production. This is in agreement with the findings of other groups who have found similar effects with excess L-arginine (1, 1a) and suggests that NO production, not L-arginine depletion, is the event which leads to decreased infectivity of chlamydial progeny at 48 and 72 h postinfection.

The mechanism through which nitric oxide synthesis affects chlamydial infectivity in McCoy cells is not yet understood and is the subject of our current investigations. Nitric oxide may kill chlamydiae, or it may inhibit chlamydial growth. Growth inhibition could occur in several ways, e.g., inhibition of EB-to-RB transformation, which would stop replication, or inhibition of RB-to-EB transformation, which would inhibit infectivity despite replication. Nitric oxide may also in some way induce chlamydiae to become dormant. Our studies using fluorescein-labelled monoclonal antichlamydial LPS antibodies to observe the development of chlamydial inclusions in McCoy cells treated with IFN indicate that both the size and the number of chlamydial inclusions present in this treatment group are markedly reduced compared with those of either non-IFN-treated McCoy cells or McCoy cells treated with IFN plus MLA which have received an identical MOI (data not shown). This finding could be compatible with either killing of chlamydiae or inhibition of chlamydial replication by NO synthesis induced in McCoy cells. The IFU determinations in Fig. 2 indicate that infectious progeny present 72 h after infection in the McCoy cells treated with 25 and 50 U of IFN-γ per ml have approximately 10^4 IFU present, compared with the initial inoculum of 5×10^5 IFU used to infect these cells. This decrease of 1 $\log_{10}$ unit in recoverable infectious progeny compared with the starting inoculum could be compatible with both modest chlamydial killing and pronounced inhibition of chlamydial growth.

Nitric oxide could have direct effects on chlamydiae to be chlamydiacidal or chlamydiastatic, as has been demonstrated with leishmanias, which are susceptible to direct effects of NO (27, 35). A potential chlamydial target of NO would include chlamydial enzymes necessary for chlamydial DNA replication, such as ribonucleotide reductase (40a), an enzyme which is known to be susceptible to the effects of NO in other cell types (33a). NO may also mediate its antichlamydial effects by affecting the host McCoy cells. In studies of macrophage killing of tumor cells induced by IFN-γ, nitric oxide production is associated with tumoricidal activity through its ability to form complexes with iron-sulfur complexes of redox enzymes (17, 18, 24, 26, 27, 32, 38). Additionally, nitric oxide has a known effect on mitochondrial respiration which may limit the ability of host cells to produce ATP (18, 24, 27, 41). If host cell ATP production is significantly decreased by NO synthesis in our model, chlamydial growth, which is dependent on ATP parasitism, could be impaired significantly. Finally, nitric oxide could affect both host cells and chlamydiae directly. We do not know at this time whether a certain threshold level of NO production is required for antichlamydial activity, as other investigators have suggested for leishmanias (34).

Our data and the data of previous investigators indicate that intracellular pathogens may provide second signals to cells which have been primed with IFN-γ, leading to control of parasitism through NO production. For chlamydiae, it will be extremely important to identify the second signal provided by chlamydiae. For example, if the molecule(s) providing a second signal is included in a chlamydial vaccine, neutralizing antibodies to the second signal could theoretically block the ability of host cells to activate the nitric oxide pathway, which we have shown to have a potent inhibitory effect on chlamydial proliferation in this murine cell line.

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